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ORIGINAL ARTICLE

Valsartan improves L-NAME-exacerbated cardiac fibrosis with TGF-β inhibition and apoptosis induction in spontaneously hypertensive rats

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Angiotensin II receptor blocker

Summary This study was designed to investigate whether chronic angiotensin II type 1 receptor blockade inhibits ventricular interstitial fibrosis with the induction of programmed cell death (apoptosis) in prolonged nitric oxide synthase (NOS) inhibition using N^G-nitro-L-arginine methyl ester (L-NAME) in spontaneously hypertensive rats (SHR). Four groups of 20-week-old male SHR were studied for 3 weeks: the control group; the L-NAME group given 80 mg/L L-NAME in drinking water; and the groups given 1 or 30 mg/(kg day) of valsartan, respectively, with L-NAME. The L-NAME group showed marked cardiac tissue injuries with elevated blood pressure such as interstitial fibrosis, intimal thickening of small arteries, and myocardial necrosis. Caspase-3, an apoptosis inducer, immunoreactivity was increased in interstitial cells, and the tissue RNA expression of transforming growth factor-β_1 (TGF-β_1) was also increased in the L-NAME group. Low-dose valsartan treatment did not affect blood pressure or cardiac weight but alleviated the L-NAME-induced interstitial fibrosis with increased mRNA level of caspase-3 in interstitial fibroblasts. High-dose valsartan significantly lowered blood pressure and decreased the mRNA levels of caspase-3 and TGF-β_1. These data suggest that low-dose valsartan inhibits interstitial fibrosis by promoting apoptosis of the fibroblasts without blood pressure changes, which may provide the TGF-β_1 inhibition in the development of interstitial fibrosis in severe hypertension rat model.

Introduction

Tissue angiotensin II (Ang II) plays an important role in the development of hypertensive cardiovascular organ injury. A number of studies have

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demonstrated that inhibitors of the renin–angiotensin–aldosterone system (RAAS) such as angiotensin-converting enzyme (ACE) inhibitors and angiotensin II type 1 receptor blockers (ARBs) are effective in protecting the cardiovascular system from hypertensive injuries by inhibiting tissue Ang II [1,2]. In spontaneously hypertensive rats (SHR) given N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase (NOS), we have observed the development of marked hypertensive organ injuries such as arteriosclerosis, nephrosclerosis, and cardiac remodeling with the enhancement of RAAS [3–5]. We have previously demonstrated the protective effects of ACE inhibitors and ARBs against nephrosclerosis [6,7]. Therefore, it seems intriguing to evaluate the therapeutic effects of ARBs on the development of cardiac remodeling in SHR given L-NAME.

In the progression of hypertensive cardiac remodeling, occurrence of interstitial fibrosis and disappearance of cells by apoptosis are also the indispensable process in addition to the proliferation and hypertrophy of cardiovascular cells [8,9]. Components of RAAS in the cardiovascular tissue such as Ang II and aldosterone induce transforming growth factor β_1 (TGF-β_1). TGF-β_1 is produced in cells such as cardiocytes, vascular smooth muscle cells, and fibroblasts and promotes fibrosis of cardiovascular tissues [10,11]. With regard to the apoptosis of cardiovascular cells, the chain reaction of the members of caspase protease family leads cells to develop apoptosis [12]. Especially, the role of caspase-3 is considered to be pivotal in the cascade to cellular apoptosis [13].

In this study, we examined the effects of valsartan, an ARB, on the tissue remodeling of left ventricle in SHR given L-NAME and assessed the involvement of cardiac expression of TGF-β_1 and caspase-3 in its therapeutic effects.

Methods

Forty-six 17-week-old male SHR (Charles River Laboratories, Tokyo, Japan) were maintained in a light controlled room at room temperature. All experiments have been approved by our institutional animal care committee. The rats were divided into 4 experimental groups: group 1 rats (control, $n = 13$) were given tap water and a regular chow containing 0.3% NaCl for 3 weeks; group 2 rats (L-NAME, $n = 9$) were given L-NAME (Sigma Chemical Co., St. Louis, MO, USA) in their drinking water (50 mg/L) for 3 weeks; group 3 rats (L-VAL, $n = 12$) were administered the same dose of L-NAME and valsartan (1 mg/(kg day) by gavage), for 3 weeks; group 4 rats (H-VAL, $n = 12$) were administered the same dose of L-NAME and valsartan (30 mg/(kg day) by gavage) for 3 weeks. Systolic blood pressure was measured by the tail-cuff method (Apollo 179, Isis, Osaka, Japan). After 3 weeks, the rats were killed under pentobarbital anesthesia (30 mg/kg, i.p.). The heart was perfused and fixed with saline and neutral-buffered 8% paraformaldehyde solution. Then, the left cardiac ventricle was excised and weighed.

The left ventricle was embedded in paraffin, and 2-μm horizontal sections were cut for light microscopic histological examination. These sections were stained with hematoxylin–eosin, periodic acid-Schiff (PAS), and Masson trichrome. Histological examination was conducted in a blinded manner. Fibrosis of the left ventricular wall was evaluated in sections stained with Masson trichrome. The area stained in blue with aniline was quantified in 10 randomly selected high-power fields (200×) using a computer system (Image Quest, Hamamatsu Photonics; Hamamatsu and MacScope, Mitani Co., Fukui, Japan), and the average percent value was used for comparison. Five-μm sections were prepared for immunohistological studies. After deparafinization, immunostaining of caspase-3 was carried out using the specific mouse monoclonal antibody (Immunotech, Marseille, France) at dilution of 1:1000 and the streptavidin/biotin immunoperoxidase method (LSAB kit, DAKO Japan, Tokyo, Japan). The percentage of the stained area in the interstitium was quantified using the image analysis computer system. The sections were also stained by terminal deoxynucleotidil transferase-mediated dUTP nick end labeling (TUNEL) method using ApoRag Peroxidase In Situ Apoptosis Detection Kit (Intergen Company, New York, NY, USA) to detect apoptotic nuclei with internucleosomal DNA fragmentation.

The total RNA of specimens was extracted from the left ventricular tissue using TRIzol reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's procedure. The first-strand cDNA was made from the total RNA using the SuperScript preamplification system (Life Technologies) with random hexamers. Excess oligomers were removed using a centrifugal filter, Microcon YM-10 (Millipore Co., Bedford, MA, USA). Real-time PCR analysis, using the ABI Prism 7700 Sequence Detection System (PE Biosystems, Foster City, CA, USA), is a method that provides reproducible quantitative PCR [14]. Cleavage of the sequence-specific probe (TaqMan Probe) by 5′ nuclease activity of the TaqDNA polymerase releases the reporter dye, resulting in an increase in emission at the corresponding wavelengths. With each cycle, the

fluorescence intensity of additional reporter dye molecules is monitored by the system. Threshold cycles were selected in the line in which all samples were in logarithmic phase. The quantity of PCR products was calculated from the threshold cycle value. This real-time detection generates quantitative data based on PCR at early cycles when PCR fidelity is highest. TGF-β_1 and caspase-3 RNA levels were quantified as the ratios to β-actin using this real-time PCR system. All oligonucleotides were obtained by chemical synthesis using the PerSeptive 8900 (PE Biosystems). TaqMan probe which was modified with fluorescence (purchased from Grener Japan, Inc., Tokyo, Japan). The nucleotide sequences of the PCR primers and TaqMan probes were as follows:

- TGF-β_1
  - o forward primer: 5′-cgcctgcagagattcaagtcaa-3′
  - o reverse primer: 5′-gtcggttcatgtcatggatggt-3′
  - o TaqMan probe: 5′-agtggctgaaccaaggagacggaataca-3′
- Caspase-3
  - o forward primer: 5′-gtgaagaaattatggaattgatg-3′
  - o reverse primer: 5′-gtagtcgcctgtgaagaaactag-3′
  - o TaqMan probe: 5′-gattctaagtcatggagatgaaggag-3′
- β-actin
  - o forward primer: 5′-cgtgaaaagatgacccagatca-3′
  - o reverse primer: 5′-acacagcctggatggctacgta-3′
  - o TaqMan probe: 5′-tttgagaccttcaacaccccagcca-3′

One-way ANOVA, followed by Duncan's multiple range test [15], was performed to test for between-group significance. All data are expressed as the mean ± S.E.M. A probability level of <5% was considered to indicate statistical significance.

Results

Table 1 lists the data of measured variables in the four groups of rats at the end of the 3-week treatment period in this study. As we have previously reported [3–7], the L-NAME group showed blunted body weight increase as compared with the control group. The low-dose and the high-dose valsartan prevented this body weight reduction in SHR given L-NAME. The L-NAME treatment accelerated the development of hypertension in SHR. The high-dose valsartan alleviated this blood pressure increase in SHR given L-NAME, while the low-dose valsartan did not significantly affect blood pressure as compared with the L-NAME group. The left ventricular weight was not significantly different between the control and the L-NAME groups. Either the low- or the high-dose valsartan did not significantly change the left ventricular weight of SHR given L-NAME.

The left panel of Fig. 1 compares the percentage of fibrosis area in the left ventricle. As compared with the control group, the L-NAME group showed markedly increased fibrosis area in the left ventricle. The treatment with valsartan reduced this cardiac fibrosis induced by L-NAME in a dose-dependent manner. Fig. 2 shows the representative micrographs of left ventricular tissue sections stained with Masson trichrome in each group. The right panel of Fig. 1 presents the percentage of interstitial area stained with anti-caspase-3 antibody. The caspase-3 immunostaining area was significantly increased in the L-NAME group as compared with the control group. The caspase-3 positive area was further increased in the L-VAL and the H-VAL groups. Fig. 3 shows the representative micrographs of caspase-3 immunostaining in the four groups. In the L-VAL and the H-VAL groups, interstitial fibrocytes were positively stained with anti-caspase-3 antibody.

The left panel of Fig. 4 shows the mRNA expression of caspase-3 in the left ventricular tissue. In accordance with the results of immunohistological study, the caspase-3 mRNA expression was higher in the L-NAME group and further higher in the L-VAL and the H-VAL groups than in the control group. As shown in the right panel of Fig. 2, the mRNA expression of TGF-β_1 in the left ventricular tissue was significantly increased in the L-NAME group as compared with the control group. Although low-dose valsartan did not significantly affect this increased TGF-β_1 mRNA in SHR given L-NAME, high-dose valsartan treatment markedly reduced the left ventricular TGF-β_1 mRNA expression to the level comparable to the control group.

Fig. 5 shows the percentage of TUNEL-positive nuclei in the fibrotic area of left ventricular tissue. The TUNEL-positive nuclei were significantly increased in the L-NAME group as compared with the control group. The percentage was further increased in the L-VAL and the H-VAL groups. Fig. 6 presents the representative micrographs of left ventricular tissue sections of TUNEL staining. The nuclei of perivascular fibrocytes were positively stained by the TUNEL method.

Discussion

Hypertension is a major risk factor for cardiovascular organ injuries. In addition, cardiovascular risk factors such as aging, dyslipidemia, and diabetes mellitus cause endothelial dysfunction and decreased NO production which further promote

Table 1 Body weight, blood pressure, and cardiac weight of rats at the end of 3-week treatment period

Indices	Control ($n = 13$)	L-NAME ($n = 9$)	L-Val ($n = 12$)	H-Val ($n = 12$)
Body weight (g)	358 ± 5	262 ± 13**	325 ± 7††	327 ± 5††
Systolic blood pressure (mmHg)	227 ± 5	276 ± 11**	265 ± 7	242 ± 5†
Left ventricular weight (g/kg)	3.25 ± 0.18	3.52 ± 0.21	3.28 ± 0.16	3.53 ± 0.19

Data are mean ± S.E.M. Values of left ventricular weight were related to unit body weight. **$p < 0.01$ vs. control; †$p < 0.05$, ††$p < 0.01$ vs. L-NAME.

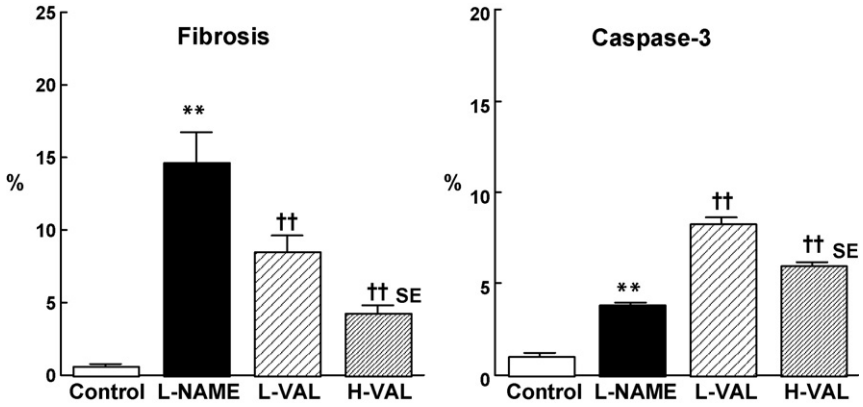

Figure 1 Effects of L-NAME and valsartan treatment on interstitial fibrosis (left panel) and caspase-3 immunostaining (right panel) in left ventricular tissue. **$p < 0.01$ vs. control; ††$p < 0.01$ vs. L-NAME.

cardiovascular tissue injuries. In this context, SHR given L-NAME, a NOS inhibitor, is assumed to be a model of cardiovascular target organ injuries caused by multiple risk factors such as hypertension and aging. In the present study, marked fibrosis took place in the left ventricular wall of L-NAME rats as compared with the control SHR although the left ventricular weight was not significantly affected. The treatment with valsartan alleviated this cardiac fibrosis in a dose-dependent manner. In rats treated with high-dose valsartan, increased apoptosis of fibrocytes induced by caspase-3 and reduced

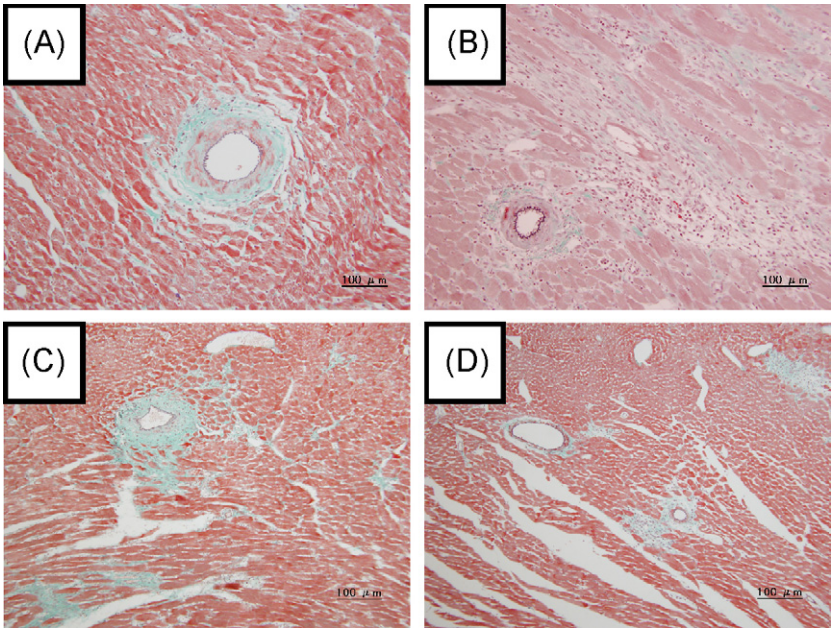

Figure 2 Representative micrographs of left ventricular tissue sections stained with Masson trichrome in the control (A), L-NAME (B), L-VAL (C), and H-VAL (D) groups.

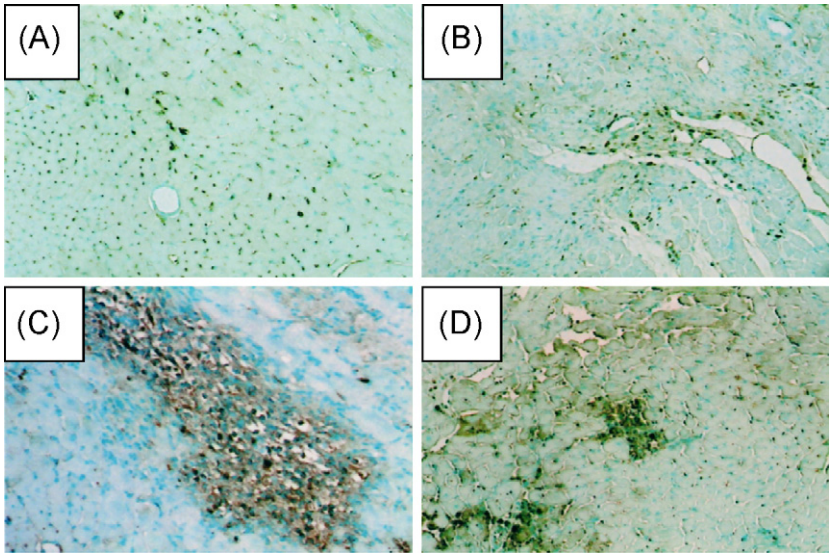

Figure 3 Representative micrographs of caspase-3 immunostaining in the control (A), L-NAME (B), L-VAL (C), and H-VAL (D) groups.

expression of TGF-β_1, in addition to blood pressure reduction, are thought to contribute to the inhibition of cardiac fibrosis. Significant improvement of cardiac fibrosis was also observed in rats treated with low-dose valsartan which did not lower blood pressure. The low-dose valsartan did not significantly reduce TGF-β_1 expression but induced caspase-3 in the left ventricular wall. Although the influence of blood pressure changes cannot be completely denied, the mechanism by which low-dose valsartan inhibits cardiac fibrosis seems related to the induction of apoptosis of fibrotic tissue cells.

We have previously reported that RAAS is enhanced in SHR given L-NAME [4,6]. Among the components of RAAS in the cardiovascular tissues, Ang II and aldosterone exert various biological actions in the cardiovascular system. It is known that actions of Ang II are mediated by type 1 and type 2 receptors. Stimulation of the Ang II type 1 receptor activates phospholipase C which generates inositol triphosphate and diacylglycerol resulting in the increase of intracellular calcium ion. Well-known actions of Ang II such as vasoconstriction and aldosterone secretion are mediated by this pathway. The promoter of TGF-β_1 gene has AP-1 binding site (phorbor ester responsible element), which is stimulated with diacyl glycerol in protein kinase C pathway [16—18]. In addition, aldosterone, secreted by the stimulation of Ang II, also increases TGF-β_1 [19]. TGF-β_1 has biphasic effects on proliferation of mesenchyme type of cells such as vascular smooth muscle cells and fibroblasts. Low dose of TGF-β_1 stimulates but high dose of TGF-β_1 inhibits proliferation of mesenchyme type of cells.

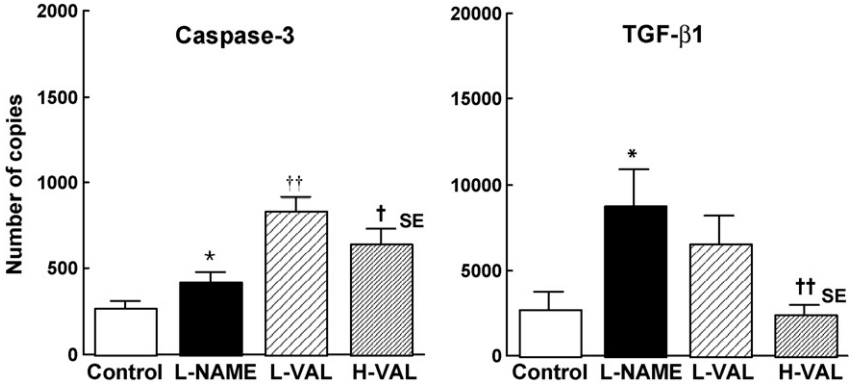

Figure 4 Effects of L-NAME and valsartan treatments on mRNA expressions of caspase-3 (left panel) and TGF-β_1 (right panel). Values in the ordinates are the numbers of mRNA copies per 10^6 β-actin mRNA copies. *$p < 0.05$ vs. control; †$p < 0.05$, ††$p < 0.01$ vs. L-NAME.

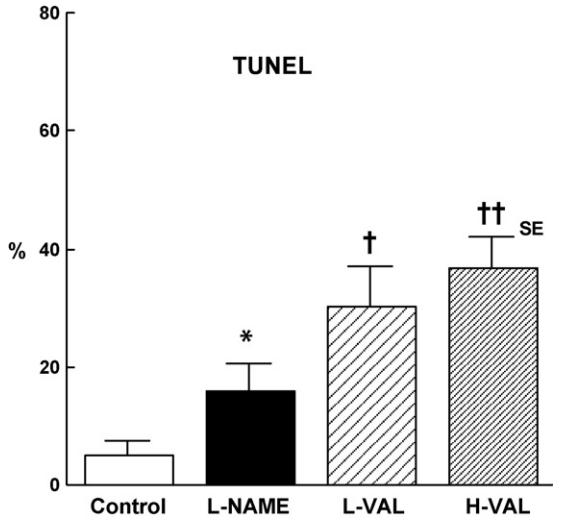

Figure 5 Effects of L-NAME and valsartan treatments on percentage of TUNEL-positive nuclei in fibrotic area of left ventricular tissue. $^{*}p < 0.05$ vs. control; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ vs. L-NAME.

TGF-β_1 increases the production of extracellular matrices and promotes the fibrosis of cardiovascular and renal tissues [10,11]. Thus, it is considered that the enhanced RAAS in the cardiovascular tissues of SHR given L-NAME contributes to the progression of cardiac fibrosis and the high-dose valsartan alleviated the cardiac fibrosis by blocking these pathways downstream of the Ang II type 1 receptor.

As compared with the Ang II type 1 receptor, actions of Ang II mediated by the type 2 receptor have not been clarified in detail. However, contrary to the actions of Ang II type 1 receptor, the stimulation of type 2 receptor causes vasodilation and inhibition of proliferation and hypertrophy of cardiovascular cells. It has been also reported that the overexpression of Ang II type 2 receptor inhibits the type 1 receptor expression by inducing bradykinin and NOS in vascular smooth muscle cells [20,21]. In addition, the Ang II type 2 receptor has been shown to elicit apoptosis of cells [22,23]. It is naturally assumed that the blocking of Ang II type 1 receptor by ARB facilitates binding of Ang II to type 2 receptors. In the present study, the cardiac expression of caspase-3 was increased in rats treated with the ARB, valsartan. Therefore, it is speculated that the increased stimulation of Ang II type 2 receptor by type 1 receptor blockade induced apoptosis of cardiac fibroblasts resulting in the inhibition of cardiac fibrosis.

Apoptosis, a morphological form of programmed cell death, is an indispensable process for the control of cell populations in various tissues and organs. It has been indicated that the occurrence of cellular apoptosis is increased in the injured heart and is supposed to have a role in the deletion of tissue volume associated with cardiac remodeling [24,25]. In the healing process after myocardial infarction, the deletion by apoptosis of damaged and nonfunctioning cardiac cells, infiltrating inflammatory

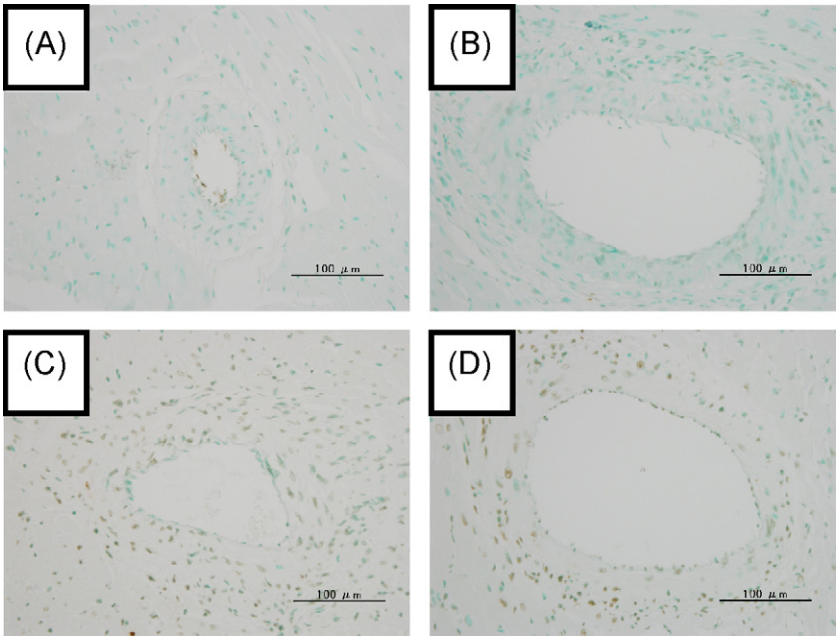

Figure 6 Representative micrographs of TUNEL-staining in the control (A), L-NAME (B), L-VAL (C), and H-VAL (D) groups.

cells, and fibroblasts is beneficial for the resolution of infarcted tissue and the reduction of fibrosis [26]. We have previously reported that SHR given L-NAME develop coronary arterial injury and myocardial infarction and fibrosis [5]. In the present study, valsartan reduced cardiac fibrosis in SHR given L-NAME and the effect may be partly mediated by the stimulation of Ang II type 2 receptor resulting in the induction of apoptosis in fibrosing tissue cells. Such beneficial effect of AngII type 2 receptor-mediated signal transduction seems to occur when the tissue injuries exist in the heart considering that the expression of Ang II type 2 receptor has been shown to increase in hypertrophied, infarcted, or failing hearts [27,28]. The increases in caspase-3 expression and apoptotic nuclei did not differ between the L-VAL and the H-VAL groups. Ang II displaced by low-dose valsartan occupies the cardiac Ang II type 2 receptors; however, the results of this study do not substantially provide information as to the lack of dose-dependency in the induction of fibrocyte apoptosis by valsartan.

The physiological and pathological roles of Ang II type 2 receptor have been investigated in experimental studies using the Ang II type 2 receptor gene knock-out mice. These gene knock-out mice have been shown to develop increased perivascular fibrosis in response to L-NAME administration and aggravation of cardiac remodeling after myocardial infarction [29,30]. In addition, it has been also reported that an ARB failed to bring about attenuation of post-myocardial infarction remodeling and amelioration of heart failure in the Ang II type 2 receptor gene knock-out mice [31]. These findings are in line with the results of the present study and indicate that the stimulation of Ang II type 2 receptor counteracts the development of cardiac remodeling and thus the cardioprotective effects of ARB are partly mediated by this receptor.

In conclusion, the ARB valsartan alleviates the development of cardiac fibrosis in SHR given L-NAME which is a model of hypertensive cardiovascular organ injury. In addition to the hypotensive effect, inhibition of TGF-β_1 expression and induction of fibrosing tissue cell apoptosis are involved in this protective effect of valsartan against cardiac remodeling. The results of the present study seem to provide information as to the mechanism of the cardioprotective effects of an ARB.

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